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(54) Title: INHIBITION OF CELL PROLIFERATION BY HYDROPHOBIC PEPTIDES

(57) Abstract

The present invention is directed a method of using certain hydrophobic peptides for the inhibition of cell proliferation, wherein the peptides have the general formula: R-Xaa_1-$(Xaa)_m$-Xaa_c. The subject peptides have 2-7 amino acids such as alanine (Ala), arginine (Arg), cysteine (Cys), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), norleucine (nLeu), phenylalanine (Phe), proline (Pro), threonine (Thr), tyrosine (Tyr), tryptophan (Trp), or valine (Val). In accordance with the present invention, these peptides are potent inhibitors of cell proliferation as well as inhibitors of the synthesis of two cellular proto-oncogenes. One aspect of the present invention provides for the prevention and treatment of cancer by administration of the subject peptides. A further aspect of the present invention provides for inhibiting cell proliferation using the subject peptides in the treatment and prevention of prostatic hypertrophy, arterial occlusion (restenosis), [illegible] and smooth muscle cell diseases.

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INHIBITION OF CELL PROLIFERATION BY HYDROPHOBIC PEPTIDES

Inappropriate, inopportune or excessive cell proliferation is a major cause not only of cancer but also of heart disease. Typically a cancerous cell loses its ability to respond to cellular signals for growth restraint. Similarly, unchecked smooth muscle cell growth within the inner lining of arteries is a major cause of arteriosclerosis and of reocclusion after arterial dilation.

The present invention is directed towards the use of certain hydrophobic peptides to inhibit cellular growth, especially of smooth muscle cells and cancerous cells. Generally these hydrophobic peptides have 2-7 amino acids such as alanine (Ala), arginine (Arg), cysteine (Cys), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), norleucine (nLeu), phenylalanine (Phe), proline (Pro), threonine (Thr), tyrosine (Tyr), tryptophan (Trp) or valine (Val). In accordance with the present invention, these peptides are potent inhibitors of cell proliferation.

One aspect of the present invention provides for the prevention and treatment of cancer by administrating the subject peptides to an animal. Another aspect of the present invention relates to inhibiting cell proliferation of smooth muscle cells with the subject peptides. An additional aspect of this invention relates to the prevention of arterial occlusion in vivo using the subject peptides and methods of administration of these peptides for the prevention and treatment of arteriosclerosis.

Biologists have long recognized that control of cell proliferation is one of the most basic aspects of multicellular existence. Throughout embryological development, and through all of adult life, differentiated cells have a choice of whether to divide or not. Only if a programmed series of correct decisions is made can the organism continue to function normally. If incorrect decisions are made one cell type can replicate unchecked, thereby interfering with critical life functions and ultimately threatening the existence of the organism.

Intertwined with any discussion or study of cell proliferation is the nature and basis of cancer. This collection of horrific diseases by definition involves cells which divide when they should not, thus producing tumors. Cancer can arise by changes in differentiated cell types, resulting in cancer cells that exhibit many of the morphological and functional characteristics of their respective non-cancerous precursor cells. Cancer is common in cells that normally undergo frequent division (e.g. epithelial cells of the skin) and so the problem may not be that cancer cells divide frequently but that they lack the normal control sytems to stop unwanted cell division. Hence, in an effort to find effective drugs for cancer therapy, reagents are sought which control or inhibit cell proliferation. The peptides of the present invention can provide this control.

However, cancer is not the only disease that is exacerbated by excessive cell proliferation. Researchers also recognize that unchecked proliferation of smooth

muscle cells can lead to partial blockage of arteries, causing arteriosclerosis. Even after treatment of arteriosclerosis by balloon dilation (angioplasty) of blocked portions of an arteries, further smooth muscle cell proliferation can cause reocclusion (restenosis) of the artery. Hence, inhibition of smooth muscle cell proliferation in arteriosclerosis, or after dilation of arteries, provides a promising new treatment strategy.

Arteriosclerosis is a disease of the inner lining, or intima, of arteries leading to formation of fatty lesions on the arterial inner surfaces. The earliest stage in the development of these lesions is believed to be damage to the endothelial cells and sublying intima. Damage can be caused by physical abrasion of the endothelium, by abnormal substances in the blood, or even by the effect of the pulsating arterial pressure on the vessel wall. Once the damage has occurred, smooth muscle cells proliferate and migrate from the media (middle layer) of the arteries into the lesions. Soon thereafter, lipidic substances, especially cholesterol, begin to deposit within the proliferating muscle cells, generating plaques. In later stages, synthesis of extracellular matrix by fibroblasts and other cells infiltrating the degenerative areas causes progressive sclerosis (fibrosis) of the arteries. Calcium often precipitates with lipids to generate calcified plaques. When fibrosis and calcification occurs, the arteries become extremely hard. The hardened arteries lose most of their distensibility, and the region within and surrounding them is easily ruptured. Arteriosclerotic plaques often protrude through the

intima into the flowing blood, and the biochemical or physical changes of the plaque surface can cause blood clots to develop.

Almost half of all human beings die of arteriosclerosis. Approximately two thirds of these deaths are caused by clot formation in a coronary artery, the remaining one third occur by clot formation, or hemorrhage of vessels, in other organs of the body, especially in the brain (causing strokes).

A commonly used treatment for coronary arteriosclerosis is percutaneous transluminal coronary angioplasty, or arterial dilation. This procedure is a treatment of choice because it can enlarge a narrowed arterial passageway. However, arterial injury may occur during angioplasty and this injury can re-initiate or intensify the process of plaque formation leading to reocclusion of the artery. In fact reocclusion (restenosis) of the artery appears to be an exaggerated response to the controlled injury of angioplasty, and occurs in 30-40% of all patients receiving angioplasty. The process of restenosis is very similar to the formation of the original arteriosclerotic plaque, but occurs on a shorter time scale. Endothelial cell injury caused by angioplasty leads to platelet aggregation and shortly thereafter to activation of smooth-muscle cell proliferation. Platelets secrete platelet-derived growth factor (PDGF), which is one of the most potent cell proliferative factors for smooth muscle cells found in serum. PDGF is also a chemotactic attractant for smooth muscle cells and may be responsible for attracting smooth muscle cells from the middle layer, or media, of

the artery into the intima as well as for the initial proliferation of smooth muscle cells within the intima. However, it is thought that PDGF alone does not optimally stimulate DNA synthesis. PDGF-stimulated cells require a second group of growth factors (termed "progression factors"), to initiate DNA synthesis and cell division. PDGF alone appears to simply stimulate cells to enter a new cell cycle by causing the cell to move from the G_0 arrest state to G_1 (Fig. 1 depicts the time course of a typical mammalian cell cycle). Exposure to progression factors allows cells to move through the cell cycle by initiating DNA synthesis (S phase). A number of progression factors are known, including epidermal growth factors from platelets and somatomedin-C present in serum.

In accordance with the present invention, specific peptides are shown to be effective in the inhibition of cell proliferation, and provide new reagents for the prevention and treatment of arteriosclerosis, prostatic hypertropy and various forms of cancer. The block in cell division caused by the present peptides appears to occur prior to the S, or DNA synthesis, phase of the cell cycle. A secondary block to cell division in the G_2 or M phase of the cell cycle, is also observed when cells are exposed to the subject peptides for longer periods of time.

The subject peptides are also inhibitors of calcium-dependent thiol proteases, calpains I and II. The calpains are cytosolic cysteine proteases which are ubiquitously distributed in most cell types. The biological function of calpains is not clear. However,

calpains have been implicated in numerous processes including the re-structuring of the plasma membrane (Zaidi, et al., 1989, J. Membrane Biol. 110:209-216), the proteolysis of two important cytoskeletal proteins (Yoshida, et al., 1984, FEBS Letters 170:259-262) as well as the regulation of platelet aggregation (Fox, et al., 1983, J. Biol. Chem. 258:9973-9981) and the activation of several cellular regulatory proteins (Murachi et al., 1981, in Advances in Enzyme Regulation, G. Weber, ed., Pergamon Press, New York, 19:407-424).

The present invention is directed to a method of inhibiting animal cell proliferation, especially the inappropriate, inopportune, or excessive cell proliferation associated with cancer, arteriosclerosis, restenosis, and smooth muscle or endothelial cells. In particular, the method provides for administering a growth inhibiting amount of at least one of certain hydrophobic peptides to an animal or to cultured cells, wherein the subject pepties range from 2 to 7 amino acids and are represented by the formula:

$$R\text{-}Xaa_1\text{-}(Xaa)_m\text{-}Xaa_c$$

and further wherein

m is 0-5;

R is hydrogen, epoxysuccinyl, cholesteryl, aryl, aralkyl or acyl;

Xaa_1 and $(Xaa)_m$ are independently Ala, Arg, Ile, Leu, Lys, Met, nLeu, Phe, Pro, Thr, Tyr, Trp or Val;

Xaa_C is an amino acid from the group Ala, Arg, Cys, Ile, Leu, Lys, nLeu, Phe, Pro, Thr, Tyr, Trp, Val or the corresponding alcohol, aldehyde, epoxysuccinate, acid

halide, carbonyl halomethane or diazomethane derivative of the carboxy terminal group of said amino acid.

Specifically, the preferred peptides of this invention include shorter peptides with two or three amino acids, i.e. m being 0 or 1, and more especially include benzyloxycarbonyl-Leu-norleucinal and acetyl-Leu-Leu-norleucinal.

Moreover, the present method is useful in treating and preventing smooth muscle cell proliferation, arteriosclerosis, restenosis, especially restenosis occuring after percutaneous transluminal coronary angioplasty, and cancer and cancerous-like conditions such as prostatic hypertrophy, small cell carcinoma of the lung and some endotheliomas and sarcomas.

Another aspect of the invention provides pharmaceutical compositions containing the subject peptides with a pharmaceutically acceptable carrier for administration to an animal in accordance with the methods of this invention.

Fig. 1 depicts the phases of the mammalian cell cycle and the approximate duration of each phase.

Fig. 2 depicts the inhibitory effect of calpeptin (benzyloxycarbonyl-leucine-norleucinal) on the proliferation of vascular smooth muscle cells. The open circles depict the growth of untreated cells; the solid circles depict the growth of cells treated with calpeptin.

Fig. 3 depicts the normal distribution of DNA in cultured vascular smooth muscle cells at various times

after addition of serum to serum depleted cells. At 12 hours (T=12 hr) after serum addition most cells have a 2n DNA content (the amount of DNA normally found in resting or non-dividing cells). Addition of serum allows the cells to enter the cell cycle and to begin DNA synthesis. By 24 hours (T=24 hr) after serum addition there is an approximate equal distribution of cells with a 2n DNA content and a 4n DNA content (twice the amount of DNA as in non-dividing cells).

Fig. 4 depicts a comparison of the amount of DNA in cells exposed to calpeptin with that in cells not exposed to calpeptin. This figure demonstrates that exposure to calpeptin arrests the division of most cells prior to DNA synthesis, since most cells exposed to calpeptin have a 2n content of DNA after 24 hours of culture in the presence of serum. The top left panel provides a control of dividing cells for comparison with the other panels: a 2n DNA content is depicted by the left peak and a 4n DNA content is depicted by the right peak. Chronic exposure of cells to calpeptin allows some progression through the cell cycle to the G_2 or M phase of the cell cycle as demonstrated by a higher DNA content in cells after 2 weeks exposure to calpeptin.

Fig. 5 depicts the effect that acetyl-Leu-Leu-norleucinal (Inhibitor I), acetyl-Leu-Leu-methioninal (Inhibitor II) and calpeptin have on DNA synthesis as measured by ^{3}H-thymidine incorporation in growing smooth muscle cells. The amount of thymidine incorporated into cells treated with 10^{-5} to 10^{-4}M of calpeptin or acetyl-Leu-Leu-norleucinal is significantly less than that incorporated into control cells.

The present invention provides a method of inhibiting cell proliferation in vivo or in vitro, using a class of hydrophobic peptides which are effective for that purpose. A general formula depicting the structures of these peptides is:

$$R\text{-}Xaa_1\text{-}(Xaa)_m\text{-}Xaa_c$$

wherein:

m is 0-5;

R is hydrogen, an epoxysuccinyl, a cholesteryl, aryl, aralkyl or acyl;

Xaa_1 and Xaa_m are independently Ala, Arg, Ile, Leu, Lys, norleucine (nLeu), Phe, Pro, Thr, Tyr, Trp, or Val;

Xaa_c is an amino acid from the group Ala, Arg, Cys, Ile, Leu, Lys, nLeu, Phe, Pro, Thr, Tyr, Trp, Val or the corresponding aldehyde, alcohol, epoxysuccinate, acid halide, carbonyl halomethane, or diazomethane derivative of the carboxy terminal amino acid.

Xaa_1 is the N-terminal amino acid; Xaa_c is the C-terminal amino acid and $(Xaa)_m$ represents internal amino acids, if present. The values of m range from 0 to 5, with preferred values of m being 0 to 3. The most preferred values for m are 0 or 1.

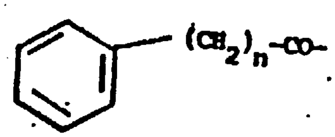
The aldehyde, alcohol, epoxysuccinate, acid halide, carbonyl halomethane, or diazomethane carboxy-terminal derivatives of Xaa_c are represented by the formulas $-CH{=}O$, $-CH_2-OH$, $-CO-\underset{O}{CH-CH}-CO_2^-$, $-CO-Y$,

$-CO-CH_2-Y$, or $-CH-N_2$ respectively. As used herein, Y is a halo group, or halide, especially Cl, Br or I. In particular, Cl or Br are preferred with Cl as the most preferred.

When R is an acyl group it has the general formula R'-CO, wherein R' is a lower alkyl or aryl group.

As used herein, the term lower alkyl refers to alkyl groups containing one to six carbon atoms. These groups may be straight-chained or branched and include such moieties as methyl, ethyl, propyl, isopropyl, n-butyl, sec-butyl, isobutyl, t-butyl, pentyl, amyl, hexyl and the like. The preferred alkyl groups are C_1-C_4 alkyl.

When R or R' is an aryl group then the term aryl, when used alone or in combination refers to an aromatic ring containing six to ten carbon atoms. Moreover, the present aryl groups include aralkyl groups (aryl groups with lower alkyl groups as ring substituents) and more specifically the groups benzyl, benzoyl, naphthyl, carboxybenzyl, benzyloxycarbonyl, or



wherein n is an integer from 0-6 and preferably 0-3. Aryl groups may have lower alkyl groups substituents at any, some or all, available ring positions. Lower alkyl group substituents are the same as those defined above.

Preferred R groups are cholesteryl, benzyloxycarbonyl, acetyl or benzoyl groups. The most

preferred R groups are acetyl, benzyloxycarbonyl and cholesteryl.

For Xaa_1 the preferred amino acids are Arg, Ile, Leu, Lys, Met, nLeu, Phe, Tyr or Val. The most preferred are Leu nLeu or Val.

Each $(Xaa)_m$ amino acid can independently be Ala, Arg, Ile, Leu, Lys, Met, nLeu, Phe, Pro, Tyr, Trp or Val. Preferred Xaa_m amino acids are Arg, Ile, Leu, Lys, Met, nLeu, Phe, Thr, Trp, Tyr, or Val. The most preferred $(Xaa)_m$ amino acids are Leu, nLeu, or Val.

Preferred Xaa_c amino acids are the aldehyde, alcohol, epoxysuccinate, acid halide, carbonyl halomethane or diazomethane derivatives of Arg, Ile, Leu, Lys, nLeu, Phe, Thr, Trp, Tyr or Val. The most preferred carboxy-terminal amino acids are the aldehyde derivatives of Leu, Lys, nLeu, Phe or Tyr.

In particular, the preferred hydrophobic peptides of this invention are calpeptin (benzyloxycarbonyl-Leu-norleucinal) and acetyl-Leu-Leu norleucinal.

The subject peptides may be chemically synthesized or isolated from a bacterial, fungal or plant source. Isolation can be by any technique used by one skilled in the art, including differential extraction, ion-exchange or gel filtration column chromatographic procedures and high pressure liquid chromatography. Chemical synthesis of the subject peptides is by any of the methods for peptide synthesis, for example by either solution or solid phase synthetic procedures such as the Merrifield procedure. Solid phase synthesis is commonly preferred for making longer peptides but many of the

short peptides (with two to four amino acids) can be made efficiently by solution synthesis.

A basic problem in peptide synthesis is one of blocking or protecting the alpha amino group from indiscriminate reaction with a carboxyl group of an undesired amino acid; an additional problem is the prevention of reactions with amino acid side chains. These undesirable side reactions are prevented by use of blocking groups that render an alpha amino group, or a side chain group, unreactive but permit the desired reaction to take place. In addition to providing protection against undesirable reactions, the blocking group must be easily removed without chemically altering the remainder of the molecule, especially the peptide linkage that has been built up during synthesis. (See generally, Morrison and Boyd, Organic Chemistry, Third Ed., Sec. 30.10 Synthesis of Peptides, pp. 1131-1133, 1983).

In practice, the strategy of maximal protection of all side chain functionalities has been routinely employed. There are at least two sets of side chain-protecting groups and the choice of one set over another is dictated by the protecting group strategy chosen for the alpha amino group. General procedures for peptide synthesis are provided in Barany et al. (1980, in The Peptides 2: 1-284, Gross E. and Meienhofer, J. eds, Academic Press, New York) and Stewart et al. (Solid Phase Peptide Synthesis, Pierce Chemical Co.).

Commonly used alpha amino protecting groups are tert-butyloxycarbonyl (Boc; cleaved by acid treatment), 9-fluorenylmethyloxycarbonyl (FMOC; removed by treatment

with a secondary amine such as piperidine) and carbobenzyoxy (Z or Cbz; removed by catalytic hydrogenation).

The alpha-amino Boc, benzyl-based side chain protection strategy relies on the principle of graduated acid lability of the protecting groups. Boc-protected amino acids are inexpensive and available in high purity. A general scheme for Boc-based peptide synthesis is depicted below.

<u>Boc-Based Peptide Synthesis</u>

$$\text{Boc-AA}_q\text{-OBz1-CH}_2\text{-CO-NH-CH}_2\text{-Resin}$$

$$\Big\downarrow \ \underline{\text{Deprotect}}$$

$$\text{Tfa-O}^- \ {}^+\text{NH}_3\text{-AA}_q\text{-oBz1-CH}_2\text{-CO-NH-CH}_2\text{-Resin}$$

$$\Big\downarrow \ \underline{\text{Neutralize}}$$

$$\text{NH}_2\text{-AA}_q\text{-oBz1-CH}_2\text{-CO-NH-CH}_2\text{-Resin}$$

$$(\text{Boc-AA})_2\text{O} \rightarrow \text{Boc-AA-OH} \qquad \Big\downarrow \ \underline{\text{Couple}}$$

$$\text{Boc-AA}_{q+1}\text{-AA}_q\text{-OBz1-CH}_2\text{-CO-NH-CH}_2\text{-Resin}$$

In the Boc-based peptide synthetic scheme depicted above, Boc-AA represents an amino acid with a Boc protecting group on its alpha amino group. The subscript q on AA depicts the number of amino acids in the peptide; q is an integer, from 1-6 in the present

invention. Tfa-O^- is trifluoroacetic acid. OBzl-CH_2-CO-NH-CH_2-Resin represents a possible coupling arm and its attachment to a solid phase resin. As described above, use of a solid phase resin is optional, depending on the size of the peptide.

The FMOC based strategy uses different mechanisms for removal of the alpha amino and side chain protecting groups: a secondary amine for the alpha amino protecting group and treatment with trifluoroacetic acid for the side chain protecting groups. An FMOC-based peptide synthesis scheme is depicted below.

<u>FMOC-Based Peptide Synthesis</u>

FMOC-AA_q-O-Bzl-O-CH_2-Resin

$\downarrow$ Deprotect

NH_2-AA_q-O-Bzl-O-CH_2-Resin

FMOC-AA-OBt $\downarrow$ Couple

HOBt

FMOC-AA_{q+1}-AA_q-OBzl-O-CH_2-Resin

An FMOC protected amino acid is represented by FMOC-AA. The O-Bzl-O-CH_2-Resin depicts an optional solid phase and its coupling arm to the synthetic peptide.

Synthesis of the present peptides need not be limited to the Boc and FMOC synthetic techniques depicted above. For example benzyloxycarbonyl-leu-norleucinal

(calpeptin) may be synthesized in solution, using benzyloxycarbonyl-leucine (Z-Leu-OH, 1) as starting material. Z-Leu-OH is first reacted with isobutylchloroformate to make a mixed anhydride as depicted below.

$$\underset{CH_2\text{-}CH(CH_3)_2}{\text{Z-NH-CH-CO-OH}} \quad + \quad Cl\text{-}CO\text{-}CH_2\text{-}CH\text{-}(CH_3)_2 \longrightarrow$$

Z-Leu-OH (1) Isobutylchloroformate

$$\underset{CH_2\text{-}CH(CH_3)_2}{\text{Z-NH-CH-CO-O-CO-}CH_2\text{-}CH(CH_3)_2}$$

Mixed Anhydride (2)

The mixed anhydride (2) is then coupled with the methyl ester of norleucine (n-Leu-OMe, 3) using a coupling reagent, for example, N-ethyl-N',N'-dimethylaminopropyl carbodiimide (WSCD).

$$\underset{CH_2\text{-}CH(CH_3)_2}{\text{Z-NH-CH-CO-O-CO-}CH_2\text{-}CH(CH_3)} \quad + \quad \underset{(CH_2)_3\text{-}CH_3}{NH_2\text{-}CH\text{-}CO\text{-}O\text{-}CH_3}$$

2 $\quad \xrightarrow{\text{WSCD}} \quad$ n-Leu-OME (3)

$$\text{Z-NH-}\underset{(CH_3)_2CH\text{-}CH_2}{\text{CH}}\text{-CO-NH-}\underset{(CH_2)_3\text{-}CH_3}{\text{CH}}\text{-CO-O-}CH_3$$

Z-Leu-nLeu-OMe (4)

The ester, Z-Leu-nLeu-OMe(4), can be saponified by reaction with a strong base to form the acid (5). Reduction with, for example sodium borohydride, yields the corresponding alcohol (6) and subsequent partial oxidation with sulfur trioxide pyridine complex, triethylamine and dimethylsulfoxide, generates the corresponding aldehyde (7). These reactions are depicted below:

$$\underset{4}{\text{Z-Leu-nLeu-OMe}} \xrightarrow{^{-}\text{OH}} \underset{5\ \text{(Acid)}}{\text{Z-Leu-nLeu-COOH}}$$

$$\underset{5}{\text{Z-Leu-nLeu-COOH}} \xrightarrow{\text{NaBH4}} \underset{6\ \text{(Alcohol)}}{\text{Z-Leu-nLeu-OH}}$$

$$\underset{6}{\text{Z-Leu-nLeu-OH}} \xrightarrow[\text{Pyridine}]{\text{Sulfur Trioxide}} \underset{7\ \text{(Aldehyde)}}{\text{Z-Leu-nLeu-H}}.$$

The above synthetic reactions illustrate general and specific techniques for synthesizing peptides, and for generating the aldehyde or alcohol derivatives from the C-terminal carboxylic acids. Substituting the C-terminal carboxylic acid for diazomethane can be accomplished by first converting the carboxylate group into a mixed anhydride using, for example, isobutyl chloroformate in tetrahydrofuran and N-ethyl morpholine. This reaction mixture can then be added to ethereal diazomethane and allowed to react overnight. The diazomethane derivatized peptide may then

be extracted with water and isolated. Chloromethane derivatized peptides may be made from the diazomethane derivative by treatment of the later with HCl in ethanol at 0°C (Green et al., 1981, J. Biol. Chem. 256: 1923-1928; Sasaki, et al., 1986, J. Biochem. 99: 173-179).

In some cases the alpha amino group of the subject peptide may be amidated by addition of a R'-CO- group, e.g., acyl group, or an epoxysuccinyl group. This can be done before, during or after peptide synthesis. Amidation may be accomplished by any of the art recognized procedures. For example, amide formation frequently involves acylation of the amine with acid chlorides, anhydrides, esters or carboxylic acids. The reaction with carboxylic acids may be of limited utility unless the acid is first activated so the -OH group becomes a good leaving group. Carbodiimides can be used to activate the carboxylic acid. Common acylating procedures for amide formation employ acid chlorides or anhydrides in pyridine.

Addition of a cholesteryl or an aryl group to the N-terminal amino acid is most easily done before the peptide is synthesized. This also prevents unwanted side reactions with non-N-terminal amino acid side groups. Blocking groups may be used on the N-terminal amino acid side group and carboxylate, if needed. By using the blocking groups described for use in peptide synthesis, the cholesteryl- or aryl-derivatized N-terminal amino acid may then be used directly in peptide synthesis. Cholesterol or any aryl group may be derivatized at a reactive position to provide a good leaving group. For

example, the C-3 alchohol of cholesterol may be derivatized with p-toluenesulfonyl chloride ($p-CH_3-C_6H_4-SO_2C_1$), thionyl chloride ($SOCl_2$) or be replaced with a halide or other group to provide a leaving group. Aryl groups with appropriate leaving groups are frequently commercially available. The appropriately blocked amino acid is then reacted with the derivatized cholesterol or aryl group under conditions which allow the leaving group to be replaced by the N-terminal amine.

After synthesis, peptides may be purified and salts may be removed by gel electrophoresis, flash chromatography with a silica gel column, or by other size selective chromatography procedures, including gel chromatography or high-pressure liquid chromatography.

The present invention is directed to peptides of the instant general formulae which have utility to inhibit ndesired cellular growth. Hence these peptides are useful, for example, in the therapy or prevention of cancer, for prevention or treatment of arterial occlusion (arteriosclerosis) and for inhibiting smooth muscle or endothelial cell proliferation.

Accordingly, the present invention provides a method of inhibiting animal cell proliferation by administering an effective amount of at least one of the hydrophobic peptides defined in accordance with this invention. An effective amount is that amount which is sufficient to inhibit cell growth or proliferation, particularly in cells which are in an inappropriate or excessive growth phase, or an amount to retard or prevent the course of a disease state.

In particular, the present method is useful for inhibiting cell proliferation whether it occurs in vivo or in vitro. When this method is used for in vivo cell inhibition, the method can include administering the subject peptides to an animal such as mammals, e.g. humans, monkeys, rabbits, mice, cows, or a veterinary animal or farm animal such as cats, dogs, chickens, turkeys, horses and the like. By in vitro cell proliferation is meant the inhibition of cultured cells, e.g. tissue culture cells or primary cell cultures obtained from a patient. Hence the method of inhibiting cultured cells can include administering, treating or co-culturing such cells in the presence of the subject peptide. For example, the peptides may be co-cultured with cells continuously or for varying periods of time.

Similarly, the subject peptides can be administered singly or in combination as dictated by the exigency of the condition. One skilled in the art can readily select and combine the subject peptides for simultaneous administration if necessary or as required by the circumstances of treatment.

The present method is particularly useful for inhibiting smooth muscle or endothelial cell proliferation. Consequently, this method provides a means of treating and preventing diseases associated with inappropriate, inopportune, or excessive cell proliferation of smooth muscle or endothelial tissues. In particular, such diseases include anteriosclerosis, restenosis and prostatic hypertrophy. The treatment and prevention of restenosis by the present method is

especially useful after percutaneous transluminal coronary angioplasty.

Moreover, the present method is particularly useful for inhibiting the proliferation of cancerous cells in an animal or in cell culture. Hence, the method provides a means of treating and preventing cancer and cancer-like diseases, e.g. benign tumors, arising from inappropriate, inopportune or excessive cell proliferation.

The administration of the subject peptides can be accomplished by any convenient route known to those skilled in the art by providing the subject peptides in an effective amount sufficient to inhibit cell proliferation as required by the exigency of the therapy. Routes of administration include oral, enteric, parenteral, intravenous, intramuscular, intrapericardial, intranasal and topical. The amount of peptide delivered varies by route and can be determined by one skilled in the art in accordance with the guidelines provided herein, especially as relates to pharmaceutical compositions.

The present peptides inhibit cellular proliferation both in vitro and in vivo. Cultured cells can be used to test for the effects of the present peptides on the rate of cell division. The rate of cell division can be assessed by exposing cells to the subject peptides, then counting cell number as a function of time and comparing to controls. Addition of the subject peptides to the cell culture medium causes a significant decrease in cell growth. Cells grown without the subject peptides increase in number by almost 10-fold in four

days. Cells grown in the presence of the subject peptides show little or no increase in number over a similar time period.

The rate of DNA synthesis can be determined by observing the incorporation of labeled nucleotide into cellular DNA. A nucleotide may be radioactively labeled. Alternatively, a labeled nucleotide may be a nucleotide analog that is readily incorporated into DNA and is detected by a second detector molecule. One example of a nucleotide analog is bromodeoxyuridine (BUdr) which may be detected by, for example, fluorescently labeled antibodies directed against bromodeoxyuridine. Total DNA content may be assessed, for example, by using propidium iodide.

To assess which stage of the cell cycle is affected by the present antiproliferative agents, cells may be synchronized or reversibly arrested in the G_o, or non-growing stage, of the cell cycle. Synchronization can be achieved by any of a number of reagents known to one skilled in the art, or simply by replacement of the culture medium with serum-free medium for 2 or more days. To initiate synchronized cell growth the cells may be rinsed with rich medium containing no cell cycle arresting reagent and then allowed to grow in normal, serum containing medium. To test the affect that the present peptides have on the growth of synchronized cells, the peptides may be added to the growth medium at various times after removal of the cell cycle arresting reagent or after addition of serum to the medium, i.e., after releasing the cell cycle arrest.

To assess the cell cycle stage of the cells, flow cytometry can be used to sort cells on the basis of DNA content. Cells which have incorporated more than a normal 2n amount of labeled or analog nucleotide can be distinguished, counted and sorted from cells with only a normal 2n DNA content. DNA content is a reflection of a cell's stage in the cell cycle. A 2n DNA content is the normal content for a cell which is not synthesizing DNA. Hence a cell with a 2n DNA content may be a non-growing cell, or a cell which has just divided but not yet begun DNA synthesis for another round of cell division. A 4n DNA content indicates that the cell has finished duplicating its normal DNA content but has not yet physically divided into two daughter cells. A DNA content between 2n and 4n indicates a cell is in the process of DNA synthesis.

Cells exposed to the subject peptides for short periods of time do not progress through the DNA synthesis (S) phase of the cell cycle and hence have a 2n DNA content long after cell cycle synchronization. However, when exposed to the present peptides for up to 2 weeks some cells may undergo DNA synthesis (i.e., have a DNA content greater than 2n) but do <u>not</u> undergo cell division, indicating that the present peptides can cause a further block in the cycle at the G_2 or M phase.

In a further analysis of the biochemistry of cells exposed to the peptides of this invention, the present invention demonstrates that transcription of two known cellular proto-oncogenes, <u>c-fos</u> and <u>c-myc</u>, are depressed relative to that observed in cells not exposed to the present peptides. The <u>c-fos</u> and <u>c-myc</u> gene

products are believed to act as transcription factors having a role in transformation of a cell to a malignant phenotype. Hence, the present peptides are useful not only for depressing cell growth but also for depressing the production of factors that can lead to malignant cancer.

The active ingredients of the present pharmaceutical compositions include the present peptides which exhibit antiproliferative activity when administered in therapeutic amounts from about 0.1 mg to about 2000 mg per kg of body weight per day and preferably in amounts of from about 1.0 to 100 mg per kg of body weight. Localized administration of a 1.0 to 1000 micromolar solutions of the subject peptides is preferred. One skilled in the art can adjust the dosage regimen to provide the optimum therapeutic response. For example, one daily dose may be administered or several divided doses may be given and the doses may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. The active compound may be administered in a convenient manner such as by an intravenous, intraparacardial, oral, intramuscular, intradermal, or subcutaneous route. The active compounds may also be administered parenterally or intraperitoneally. Intravenous or intrapericardial administration is preferred.

Depending on the route of administration, the active ingredients of the subject pharmaceutical composition may be coated in a material to protect the ingredients from the action of enzymes, acids or other natural products. Dispersions can be prepared in

glycerol, liquid polyethylene glycols, oils and in mixtures thereof. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

The pharmaceutical preparations suitable for injection include sterile liposomal suspensions, aqueous solutions or dispersions, as well as sterile powders for extemporaneous preparation of injectable solutions or dispersions. In all cases the preparation must be sterile and must be fluid to the extent that it is easily syringable. A preparation must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of microbial action can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it is preferable to include isotonic agents, for example, sugars or sodium chloride.

Preferred carriers are those which protect the active compound against rapid elimination from the body, such as controlled release formulations, including implants and microencapsulated delivery systems.

Biodegradable, biocompatible polymers can be used, such as polyanhydrides, polyglycolic acid, collagen and polylactic acid.

Liposomal carriers are also contemplated as preferred carriers by the present invention. In addition to the subject peptides, liposome carriers may incorporate within them agents which help target the subject peptides to the appropriate cell type, e.g. antibodies directed against membrane proteins found only on a specific cell type. Liposomal formulations may be prepared by dissolving appropriate lipids in an inorganic solvent which is subsequently evaporated to generate a thin film of dried lipid on the surface of a container. Appropriate lipids may include stearoyl phosphatidyl ethanolamine, stearoyl phosphatidyl choline, arachadoyl phosphatidyl choline and cholesterol. An aqueous solution of the active compound, with the desired additional carriers or agents as described above, is then introduced into the container. The container is then swirled to free the lipids from the sides of the container and to disperse lipid aggregates, thereby forming the liposomal suspension. Methods for preparation of liposomal formuations will be apparent to those skilled in the art.

Sterile injectable solutions may also be prepared by incorporating the active compounds in the required amount and in the appropriate solvent with various of the other ingredients enumerated above, followed by filter sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains

the basic dispersion medium and the required other ingredients (enumerated above). In the case of sterile powders for the preparation of sterile injectable solutions, the preferred method of preparation is vacuum drying. This freeze-drying technique yields a powder of the active ingredient with any additional desired ingredients from the previously sterile-filtered solution.

When the present peptides are suitably protected they may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or they may be enclosed in hard or soft gelatin capsule. They may also be compressed into tablets, or incorporated directly into a food which is part of the diet. For oral therapeutic administration, the active peptide may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5% to about 80% of the weight of the unit. The amount of active compound in such therapeutically useful compositions is such that a suitable dosage is obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral unit dosage form contains between about 1 µg and 2000 mg of active compound.

The tablets, troches, pills, capsules and the like may also contain the following: a binder such as gum tragacanth, acacia, corn starch or gelatin;

excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavoring agent such as peppermint, oil or wintergreen, or cherry flavoring. When the dosage form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the unit dosage. For instance, tablets, pills, or capsules may be coated with shellac. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavoring such as cherry or orange flavor. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound may be incorporated into sustained-release preparations and formulations.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Unit dosage form as used herein refers to physically discrete units suitable as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the novel dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of

the active material and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active material for the prevention of disease in living subjects.

The principal active peptide ingredient, is compounded for convenient and effective administration in pharmaceutically effective amounts with a suitable pharmaceutically acceptable carrier in dosage unit form as hereinbefore disclosed. A unit dosage form can, for example, contain the principal active compound in amounts ranging from 1 ug to about 2000 mg. Expressed in proportions, the active compound is generally present in from about 1 ug to about 2000 per ml of carrier. In the case of compositions containing supplementary active ingredients, the dosages are determined by reference to the usual dose and manner of administration of the said ingredients.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

The following examples further illustrate the invention.

EXAMPLE 1

Materials and Methods

Cell Culture

Bovine aortic smooth muscle cells were obtained by outgrowth from medial explants of thoracic aortae of cows within 4 hours of slaughter. Initial outgrowth as well as standard maintenance growth was in DMEM with 10% FBS added. Media were renewed every 2-3 days. All growth was in a humidified incubator equilibrated with a 5% CO_2 atmosphere. Cultures were passaged immediately prior to full confluence by brief exposure to HBSS (Hank's Balanced Salt Solution) containing trypsin (0.5 mg/ml) and EDTA (0.5 mM); all experiments were performed using cells of passage 7 or less. As a test of viability, cells were counted and assessed for trypan blue exclusion with a hemocytometer at each passage and at selected times during time course experiments, routinely showing >95% of the population to exclude trypan blue. For most subcultures and all experiments cells were plated at a density of 10,000 cells/cm^2, regardless of container.

These cells exhibited typical morphologic characteristics of vascular smooth muscle in vitro including a pattern of variably multilayered growth, and demonstrated specific immunoperoxidase staining by a monoclonal antibody selective for muscle α-actin (HHF-35) which did not react with endothelial cells, and is known not to stain fibroblasts (Tsukada, et al., 1987, Am. J. Pathol. 127:51-60).

[^{3}H] Thymidine Incorporation

[^{3}H] thymidine incorporation was used to assess cell growth. After obtaining cells as described above they were seeded in 24-well microtiter plates and allowed to attach overnight. The cultures were washed with PBS and placed in serum-free medium, consisting of DMEM with 1 µM insulin and 5 µg/ml transferrin added, for a total of 48-52 hours (more than one doubling time for actively cycling cells). During the last 6 hours of this incubation, agents were added at variable concentrations in a carrier solution consisting of 1:1 EtOH: H_2O to a final EtOH concentration of 0.5 mg per 100 ml. Serum-free medium was then removed and replaced by DMEM with 10% FBS using concentrations of agent and carrier solutions identical to those in the serum-free medium. [^{3}H] thymidine was added 18 hours after serum repletion to a concentration of 2 µCi/ml, and was incubated for 6 additional hours. At the end of the incubation, cells were released from the wells and incorporated precursor removed by washing with distilled water. Cell residues were collected on glass mesh filter by a automated cell harvester. Radioactivity was measured by liquid scintillation spectroscopy.

Flow Cytometric Assays

Cells were plated, allowed to attach, and placed in serum free media for 48-52 hours as described above. Compounds to be tested were added at the designated concentrations in the serum-free media followed by serum-containing media, also as described above. At the specified times after serum addition, these cells were harvested by trypsin/EDTA, washed with

HBSS/5% BSA, pelleted, and resuspended in PBS containing 0.6% NP-40 and 0.1 mg/ml propidium iodide, to which was added RNAse to a final concentration of 2mg/ml. Flow cytometric analyses were done using a Becton-Dickinson FACScan wherein the exciting wavelength was at 488 nm and the detecting wavelength at 585 nm. Events were gated on a fluorescence-area vs.-width map to eliminate potential clumped nuclei. Such events represented less than 3% of the total in general.

Calpeptin Synthesis

Calpeptin was synthesized using an adaptation of the protocol described by Tsujinaka et al. (1988, Biochem. Biophys. Res. Comm. 153:1201-1208). Briefly, Z-leu-OH was reacted with isobutyl chloroformate to form a mixed anhydride intermediate; this was then coupled to nLeu-OMe-HCl, followed by saponification with 1 N NaOH to yield Z-Leu-nLeu-OH. This was reduced to the alcohol with sodium borohydride, and then partially oxidized to the corresponding aldehyde with sulfur trioxide/pyridine. The overall yield starting from the original components was 26% after purification by flash chromatography with a silica gel column using initially 20% then 30% ethyl acetate in hexanes mixture. Following recrystallization from hot hexanes, the melting point was 90-93°C. Purity was confirmed by TLC in 1:1 ethyl acetate:hexanes.

EXAMPLE 2

The Present Peptides are Antiproliferative Agents for Cells in Culture

Cells were cultured as described in Example 1. To assess cell growth, either tritiated thymidine or a deoxynucleotide analog, bromodeoxyuridine (BUdr) was added to the medium; the incorporation of tritiated thymidine or of fluorescently labeled antibodies directed against bromodeoxyuridine, respectively, gave a measure of cell proliferation. Addition of propidium iodide to the medium allowed determination of the total amount of DNA. Cells were sorted by flow cytometry to distinguish populations of cells with different DNA (i.e., in this example, BUdr) contents.

Addition to the medium of benzyloxycarboxyl-Leu-norleucinal at 100 µM or acetyl-Leu-Leu-norleucinal at 50 µM caused a significant decrease in cell growth. As depicted in Fig. 2, the number of cells normally increased almost 10-fold in 4 days after serum addition but addition of calpeptin (benzyloxycarbonyl-Leu-norleucinal) resulted in almost no increase in cell number.

Removal of serum from the medium was used to synchronize the cell cycle of cultured cells (Fig. 3), thereby allowing an assessment of which phase of the cell cycle was affected by the compounds tested. As depicted in Fig. 1, cells normally require about 24 hr to progress through the cell cycle. After serum depletion almost all cells are arrested in the G_o/G_1 phase with a 2n DNA content (a normal, non-dividing amount of DNA). As depicted in Fig. 3, untreated cells exhibit little

increase in DNA content at 12 hr after addition of serum. However, by 18 hr a significant number of untreated cells have more than a 2n DNA content, indicating that DNA synthesis is progressing. By 21 hr, approximate equal numbers of cells have a 2n or 4n DNA content, while the remaining cells have a DNA content between 2n and 4n. By 24 hr almost all untreated cells have a 4n or 2n DNA content. Cells with a 4n DNA content are likely undergo cell division shortly, while those with a 2n DNA content may have just undergone cell division.

Fig. 4 depicts the effect of calpeptin on the DNA content of cells. In the absence of calpeptin and 24 hr after serum addition to serum-depleted cells, more cells have a 4n DNA than a 2n DNA content, indicating that significant DNA synthesis is occurring in these cells. However, cells exposed to calpeptin do not progress through the DNA synthesis phase of the cell cycle, as demonstrated by the 2n DNA content of almost all calpeptin exposed cells in Fig. 4. By 2 weeks after serum addition most non-exposed cells have a 2n DNA content indicating that they have stopped growing. Many calpeptin-exposed cells, on the other hand, have a 4n DNA content at 2 weeks after serum addition, indicating that some cell cycle progression has occurred, and that an additional block has occurred in these cells at the G_2 or M phase of the cell cycle. No increase in aneuploidy of DNA content (over 4n) was observed relative to controls. Cell counting demonstrated that no cell growth had occurred in 2 weeks in cells exposed to calpeptin or acetyl-Leu-Leu-norleucinal and that there were less than 20% nonviable cells at any time. The EC_{50} of calpeptin

and acetyl-Leu-Leu-norleucinal is 56 and 14 μM, respectively.

Fig. 5 depicts the effects of different concentrations of calpeptin, acetyl-Leu-Leu-norleucinal (Inhibitor I of Fig. 5) and acetyl-Leu-Leu-methioninal (Inhibitor II of Fig. 5) on DNA synthesis in proliferating aortic smooth muscle cells as measured by tritiated thymidine incorporation by smooth muscle cells. Cells exposed to either calpeptin or acetyl-Leu-Leu-norleucinal incorporate significantly less tritiated thymidine than do control cells or cells exposed to a synthetic peptide which is not an anti-proliferative agent (Inhibitor II).

EXAMPLE 3

The Present Peptides Inhibit Transcription of Proto-Oncogenes

By Northern analysis of total cellular mRNA produced after addition of serum to serum depleted cells, acetyl-leucine-leucine-norleucinal caused a 4-fold decrease, relative to controls, in the expression of c-fos and c-myc, known cellular proto-oncogenes. A similar decrease in actin mRNA was observed upon addition of acetyl-leucine-leucine-norleucinal. In all cases, mRNA was obtained from the same number of cells. The production of mRNA from adenine phosphoribosyl-transferase (APRT), a housekeeping gene required for general metabolic function in all cells, was used as an internal standard against which different mRNA preparations could be compared. No decrease in APRT mRNA was seen in treated cells, relative to untreated cells. Hence, these peptides not only inhibit cell proliferation but also depress the synthesis of cellular oncogenes.

EXAMPLE 4

Prevention of Restenosis After Angioplasty by the Present Peptides

Acetyl-Leu-Leu-norleucinal has been shown to reduce proliferation of cells in culture (Example 2). To demonstrate that acetyl-Leu-Leu-norleucinal and related 0 peptides can have a similar effect in vivo, and be useful in the prevention of arteriosclerosis and restenosis after angioplasty, arteriosclerotic rabbits were treated with acetyl-Leu-Leu-norleucinal immediately after angioplasty. Prevention of restenosis after angioplasty was used as a test system for prevention of arteriosclerosis because the underlying mechanisms of restenosis are like those occurring during arteriosclerotic plaque formation.

A 50 µM solution of acetyl-Leu-Leu-norleucinal was slowly injected into 7 arteriosclerotic rabbit femoral arteries immediately following angioplasty. A porous Wolinsky catheter was used for this injection to slowly disperse the peptide into the artery for a period of 45 sec. As a control, five contralateral arteries were injected with the carrier solution containing no acetyl-Leu-Leu-norleucinal (Control 1). As a further control, 12 femoral arteries were treated only with angioplasty (Control 2). All animals were sacrificed after angiography, which was done 2 weeks after angioplasty. Arteries were sectioned into 3mm segments, stained with hematoxylin/eosin and elastin/trichrome and morphometry was performed. Results were evaluated by analysis of variance.

Table 1 summarizes the results of the study. Angiography showed that the luminal diameter of animals treated with acetyl-Leu-Leu-norleucinal was larger than in controls, i.e. there was a smaller decrease after angioplasty in treated than in non-treated animals. Similarly, histological measurements indicated that treated animals had a larger lumen area than control animals. Neointimal area was the same for all groups (0.76 ± 0.42 mm^2 for control 1 animals and 0.63 ± 0.31 mm^2 for control 2 animals; $p = 0.817$). However, the neointima/media ratio was smaller in acetyl-Leu-Leu-norleucinal treated animals due to an increased medial thickness. These data demonstrate that injection of these peptides reduces post-angioplasty restenosis while preserving the medial layer.

TABLE 1
Effect of Acetyl-Leu-Leu-norleucinal (Inhibitor 1) on Restenosis after Angioplasty

Treatment after angioplasty	Decrease in arterial lumina (by angioplasty	Luminal area (by histology)	Medial Thickness
None	0.83 ± 0.36 mm	0.54 ± 0.26 mm^2	0.38 ± 0.05
Injection with Carrier Solution	0.88 ± 0.33 mm	0.55 ± 0.19 mm^2	0.45 ± 0.14
Injection with Inhibitor 1	0.26 ± 0.27 mm	0.84 ± 0.40 mm^2	0.76 ± 0.38
Statistical significance (check)	p = 0.004	p = 0.113	p = 0.02

WHAT IS CLAIMED IS:

1. A method of inhibiting animal cell proliferation which comprises administering an effective amount of a hydrophobic peptide sufficient to inhibit cell growth, wherein said peptide has the formula:

$$R\text{-}Xaa_1\text{-}(Xaa)_m\text{-}Xaa_c$$

wherein:

m is 0-5;

R is hydrogen, epoxysuccinyl, cholesteryl, aryl, aralkyl or acyl;

Xaa_1 and $(Xaa)_m$ are independently an amino acid selected from the group consisting of Ala, Arg, Ile, Leu, Lys, Met, nLeu, Phe, Pro, Thr, Tyr, Trp and Val;

Xaa_c is an amino acid selected from the group consisting of Ala, Arg, Cys, Ile, Leu, Lys, nLeu, Phe, Pro, Thr, Tyr, Trp and Val, or the corresponding alcohol, aldehyde, epoxysuccinate, acid halide, carbonyl halomethane or diazomethane derivative of the carboxy terminal group of said amino acid.

2. The method of Claim 1 wherein m is 1.

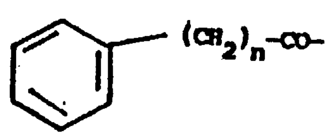
3. The method of Claim 1 wherein m is 0.

4. The method of Claim 2 or 3 wherein Xaa_1 is Leu, Val or nLeu.

5. The method of Claim 2 wherein Xaa_m is Leu or Val.

6. The method of Claim 2 or 3 wherein Xaa_c is Lys, Leu, nLeu, Phe or Tyr.

7. The method of Claim 2 or 3 wherein R is acetyl, benzyloxycarbonyl, cholesteryl, epoxysuccinyl, phenyl or



wherein n is 0 to 6.

8. The method of Claim 2 wherein the hydrophobic peptide is acetyl-Leu-Leu-norleucinal.

9. The method of Claim 3 wherein the hydrophobic peptide is benzyloxycarbonyl-Leu-norleucinal.

10. The method according to any one of Claims 1, 2, 3, 8 or 9 wherein said cell proliferation is inhibited in smooth muscle cells.

11. The method according to any one of Claims 1, 2, 3, 8 or 9 wherein said cell proliferation is inhibited for the treatment of prostatic hypertrophy.

12. The method according to Claims 1, 2, 3, 8 or 9 wherein said cell proliferation is inhibited for the treatment of arteriosclerosis.

13. The method according to any one of Claims 1, 2, 3, 8 or 9 wherein said cell proliferation is inhibited for the treatment of restenosis.

14. The method of Claim 13 wherein said restenosis follows percutaneous transluminal coronary angioplasty.

15. The method according to any one of Claims 1, 2, 3, 8 or 9 wherein said cell proliferation is inhibited for the treatment of cancerous cell proliferation.

16. A pharmaceutical composition for inhibiting cell proliferation comprising a therapeutic amount of at least one hydrophobic peptide and a pharmaceutically acceptable carrier, wherein said peptide has the formula:

$$R\text{-}Xaa_1\text{-}(Xaa)_m\text{-}Xaa_C$$

and further wherein:

m is 0-5;

R is hydrogen, epoxysuccinyl, cholesteryl, aryl, aralkyl or acyl;

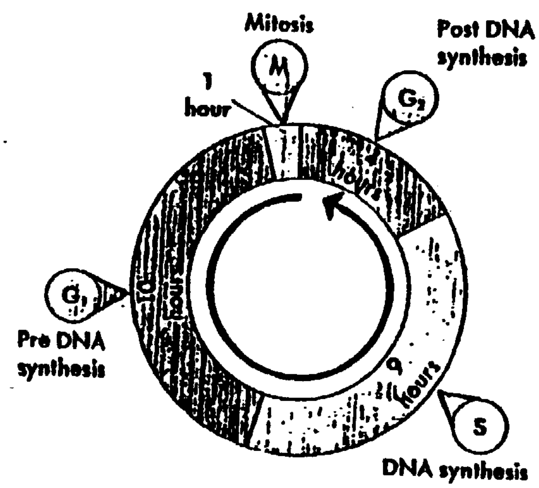
Xaa_1 and $(Xaa)_m$ are independently an amino acid selected from the group consisting of Ala, Arg, Ile, Leu, Lys, Met, nLeu, Phe, Pro, Thr, Tyr, Trp and Val;

Xaa_c is an amino acid selected from the group consisting of Ala, Arg, Cys, Ile, Leu, Lys, nLeu, Phe, Pro, Thr, Tyr, Trp and Val, or the corresponding alcohol, aldehyde, epoxysuccinate, acid halide, carbonyl halomethane or diazomethane derivative of the carboxy terminal group of said amino acid.

17. The composition of Claim 16 wherein said peptide is acetyl-Leu-Leu-norleucinal or benzyloxycarbonyl-Leu-norleucinal.

18. The composition of Claim 16 wherein said peptide is present in an amount to provide from about 0.1 mg to about 2000 mg per kilogram of body weight per day.

Figure 1


Mitosis
M
1
hour
Post DNA
synthesis
G_2
hours
G_1
10
hours
Pre DNA
synthesis
9
hours
S
DNA synthesis

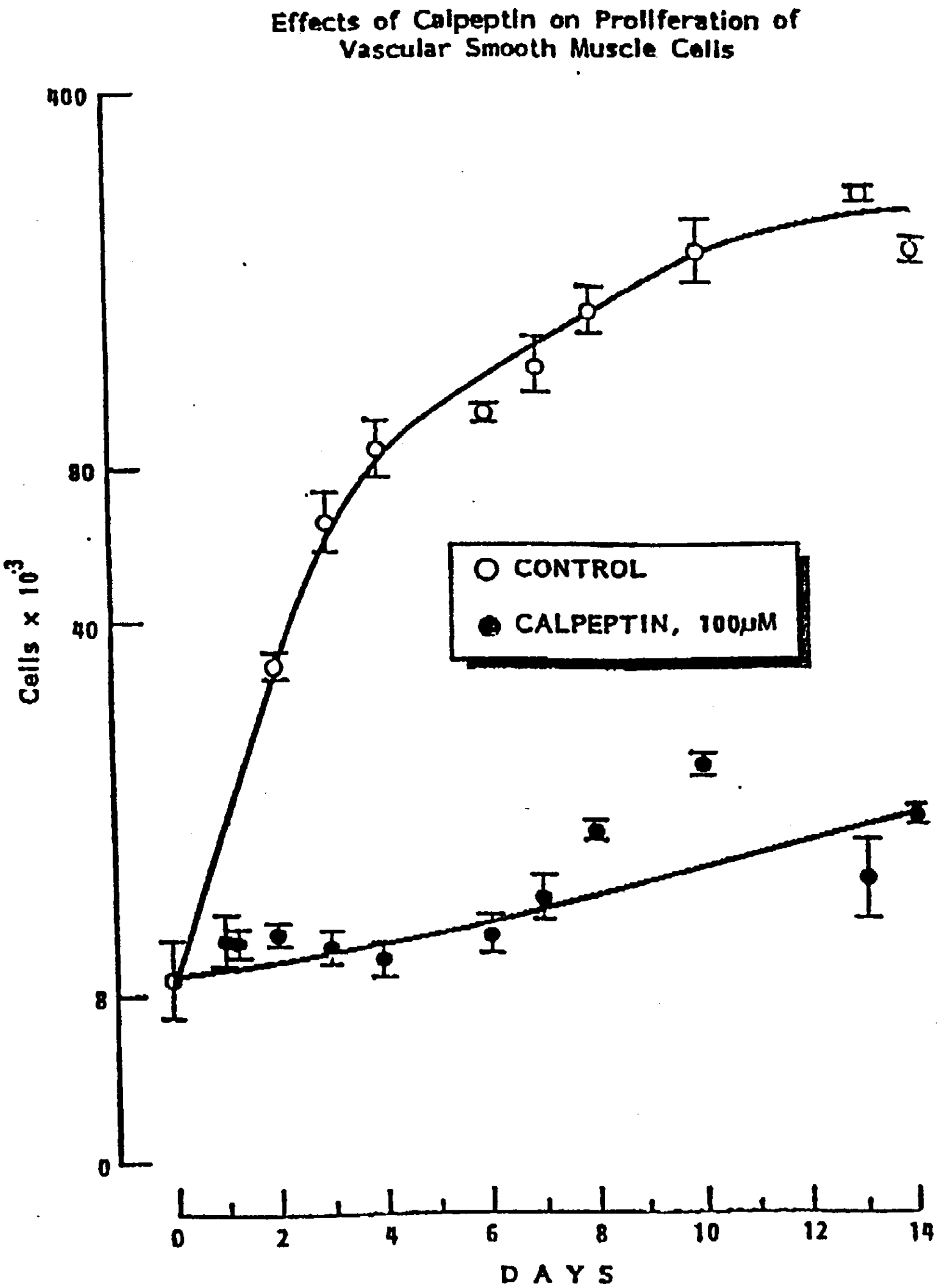

Effects of Calpeptin on Proliferation of
Vascular Smooth Muscle Cells
400
80
40
8
0
Cells x 10^3
CONTROL
CALPEPTIN, 100μM
0
2
4
6
8
10
12
14
DAYS

Figure 3

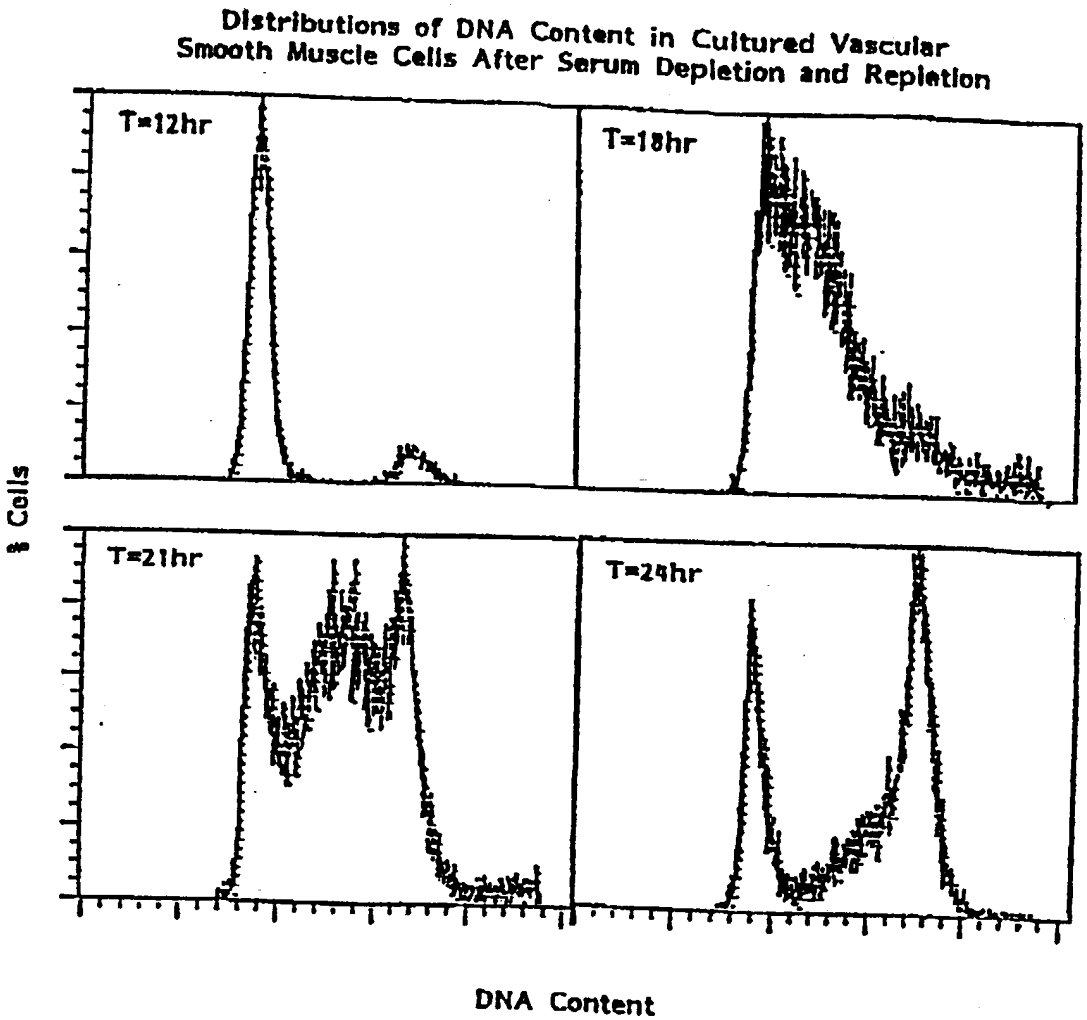

Distributions of DNA Content in Cultured Vascular Smooth Muscle Cells After Serum Depletion and Repletion
T=12hr
T=18hr
T=21hr
T=24hr
% Cells
DNA Content

Figure 4

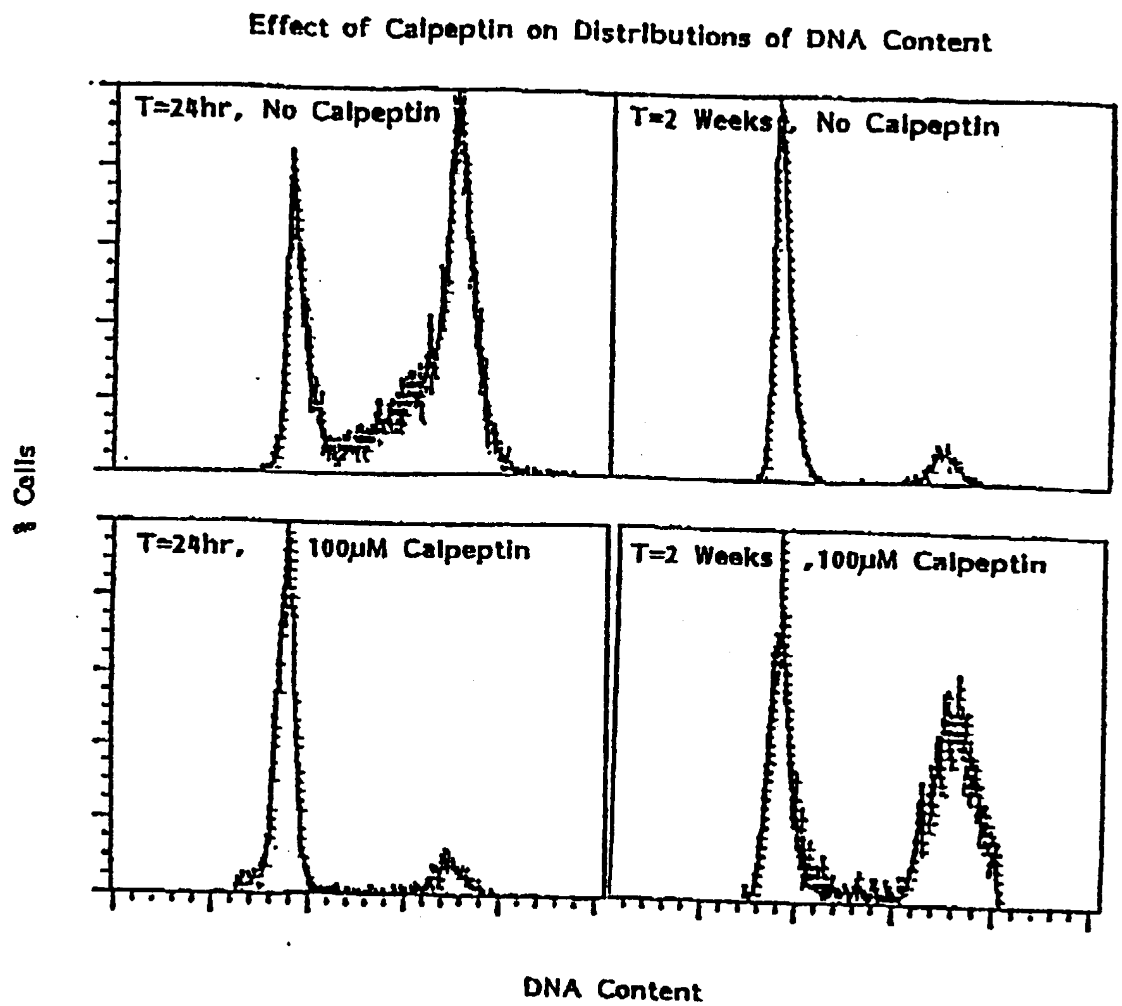
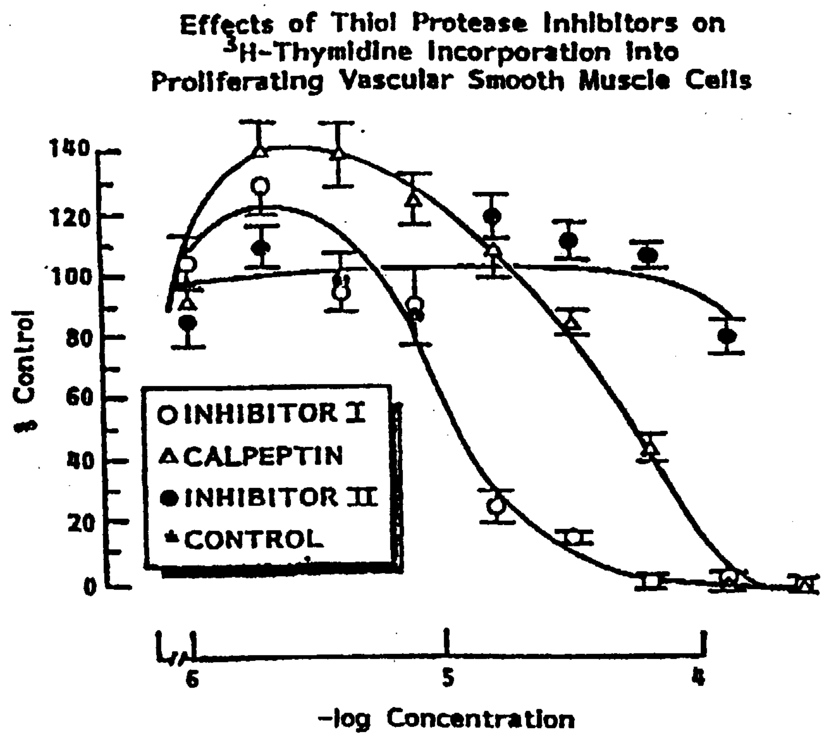

Effect of Calpeptin on Distributions of DNA Content
T=24hr, No Calpeptin
T=2 Weeks, No Calpeptin
T=24hr, 100μM Calpeptin
T=2 Weeks ,100μM Calpeptin
% Cells
DNA Content

Figure 5


Effects of Thiol Protease Inhibitors on
^{3}H-Thymidine Incorporation into
Proliferating Vascular Smooth Muscle Cells
% Control
140
120
100
80
60
40
20
0
O INHIBITOR I
△ CALPEPTIN
● INHIBITOR II
* CONTROL
6
5
4
-log Concentration

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/00905

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): A61K 37/02; C07K 5/00
US CL : 530/329,330,331; 514/17,18,19

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	530/329,330,331; 514/17,18,19

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
X/Y	US, A. 4,752,602 (Lipsky et al.) 21 June 1988, see entire document.	1,3-7,16,18/9
X/Y	EP, A, 0,383,190 (Guindon et al.) 22 August 1990, see entire document.	1,10,16,18/ 2,8,11,12,13,14,15,16-18
Y	Biochemical And Biophysical Research Communications, Volume 153, No.3, issued 30 June 1988, Tsujinaka et al. "Synthesis Of A New Cell Penetrating Calpain Inhibitor (Calpeptin)", pages 1201-1208, see tables I and II on pages 1204-1205.	16-18
Y	JP A, 61-103897 (Murachi et al.) 22 May 1986 See entire document.	16-18
Y	Blood, Volume 76, No. 12, issued 15 December 1990, Fox et al., "The Role of Calpain in Stimulus-Response Coupling: Evidence That Calpain Mediates Agonist-Induced Expression of Procagulant Activity In Platelets", pages 2510-2519, see entire article.	16-18

* Special categories of cited documents:[15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
05 MAY 1992	21 MAY 1992
International Searching Authority[1]	**Signature of Authorized Officer[20]**
ISA/US	Deborah Fruse for Bennett Celsa

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/00905

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): A61K 37/02; C07K 5/00
US CL : 530/329,330,331; 514/17,18,19

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	530/329,330,331; 514/17,18,19

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
X/Y	US, A. 4,752,602 (Lipsky et al.) 21 June 1988, see entire document.	1,3-7,16,18/9
X/Y	EP, A, 0,383,190 (Guindon et al.) 22 August 1990, see entire document.	1,10,16,18/ 2,8,11,12,13,14,15,16-18
Y	Biochemical And Biophysical Research Communications, Volume 153, No.3, issued 30 June 1988, Tsujinaka et al. "Synthesis Of A New Cell Penetrating Calpain Inhibitor (Calpeptin)", pages 1201-1208, see tables I and II on pages 1204-1205.	16-18
Y	JP A, 61-103897 (Murachi et al.) 22 May 1986 See entire document.	16-18
Y	Blood, Volume 76, No. 12, issued 15 December 1990, Fox et al., "The Role of Calpain in Stimulus-Response Coupling: Evidence That Calpain Mediates Agonist-Induced Expression of Procagulant Activity In Platelets", pages 2510-2519, see entire article.	16-18

* Special categories of cited documents:[15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Form PCT/ISA/210 (second sheet)(May 1986) 6